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ULTRASTRUCTURE OF THE SPERMATOGENIC PROCESS IN THE PENSHELL ATRINA MAURA (BIVALVIA: PINNIDAE)

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ABSTRACT

The ultrastructural features of the spermatogenic development and mature spermatozoa morphology of Atrina maura were investigated through transmission electron microscopy. The testis displays a diffuse tubular structure in which gametes develop within a concentric arrangement according to the degree of development. Within the tubules also appear Sertoli cells, which contain a large amount of lipid droplets and glycogen granules in the cytoplasm, suggesting a nutritive role in the first spermatogenesis stage (spermatogonia). Four gamete maturation stages co-occurred in the same individual: spermatogonia (4-8 um), spermato-cytes (3-4 um) (including primary and secondary), spermatids (2.5 um) and spermatozoa (- 2 um head length). The proacrosomal vesicle and flagellum occur only until the first stages of spermiogenesis, contrasting with the early formation of both structures in spermatogonia and spermatocytes in other bivalves. In A. maura, the material in the acrosomal vesicle of mature spermatozoa shows a characteristic electron density pattern, which seems to be an exclusive taxonomic feature of the family Pinnidae. However, A. maura displayed only four mitochondria in the midpiece, contrasting with five in all other species in this family.
Key words: acrosomal complex, Sertoli cells, spermatic ultrastructure, Pteriomorphia, taxonomic implications.

INTRODUCTION

Atrina maura (С. В. Sowerby I, 1835) is one of
the edible bivalve species of economic impor-
tance in Mexico. Its distribution range stretches
from Baja California Sur, Mexico (including the Gulf of California) to Peru (Coan & Valentich-Scott, 2012). It has been recognized as an abundant natural resource along temperate and tropical Mexican Pacific coasts for several decades (Vélez-Barajas & Fajardo-Leon, 1996; Ahumada-Sempoal et al., 2002). But recently, Baja California Sur fishers have witnessed a marked decline in the abundance of this species in several harvesting areas, although government officials have not reported this decline. Derived from this situation, several re-search institutions have made various attempts to produce A. maura seed in the laboratory. However, a number of unsolved questions remain about the reproductive biology of the

species, and there is no standardized culture methodology to date (Reynoso-Granados et al., 1996; Cardoza-Velasco & Maeda-Martinez, 1997; Lora-Vilchis et al., 2004).
A number of investigations have been conducted previously on the reproductive features ofA . maura, including gonad develop-ment, maturation and the reproductive cycle (Rodriguez-Jaramillo et al., 2001; Enriquez-Diaz et al., 2003; Angel-Perez et al., 2007; Angel-Dapa et al., 2010; Camacho-Mondragon et al., 2012). However, these investigations have focused on histology using light micros-copy and paraffin-embedded sections, leading to a limited interpretation of the cytological characteristics due to the intrinsically low
resolution of this technique. Studies on sper-
matic ultrastructure have proven to be a useful
source of information about additional features
for taxonomic and phylogenetic analyses in
bivalves (Franzen, 1983; Hodgson et al., 1990;

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330 CAMACHO-MONDRAGON ETAL.

Healy, 1996; Kafanov & Drozdov, 1998; Healy
et al., 2000). Similarly, these studies constitute
the framework for carrying out more special-
ized work, that is, molecular analysis and for
gamete characterization at the cell level, as
accomplished in vertebrate species (Com et al., 2003). Moreover, ultrastructural descriptions serve as an initial model from which studies comparing abnormalities caused by contami-nants or diseases may derive (Yurchenko & Vaschenko, 2010).
To date, there are no published studies on the ultrastructure of spermatogenesis to supple-ment the morphological descriptions of the gametes for A. maura, which would advance the basic understanding of the reproductive biology of this species. Furthermore, there is little information on spermatic ultrastructure for the Pinnidae family (subclass Pteriomorphia), which comprises only two genera: Pinna and Atrina (Gaulejac de et al., 1995; Healy et al., 2000; Kang et al., 2012). The only published works on this family refer to Pinna nobilis (Linnaeus, 1758) (Gaulejac de et al., 1995) and describe all the spermatogenesis stages; however, the characterization of the most important organelles, that is, the acrosomal complex, require further investigation. As regards the genus Atrina, the ultrastructure of spermatozoa has been investigated in detail for Atrina vexillum (Born, 1778) (Healy et al., 2000) and Atrina pectinata (Linnaeus, 1767) (Kang et al., 2012); in the latter, cell differen-tiation during spermatogenesis has also been analyzed.
The present study describes the ultrastruc-tural features of spermatogenesis in A. maura and emphasizes the morphological charac-teristics of spermatozoa that are considered taxonomically and phylogenetically relevant, allowing interspecific comparisons while pro-viding information on the general spermatozoa pattern in the family Pinnidae.

MATERIALS AND METHODS

Atrina maura males (> 12 cm shell length) were collected in the Ensenada de La Paz, Baja California Sur, Mexico, which are considered as adults based on the size at first maturity of this particular species in the same area (Camacho-Mondragön et al., 2012). Gonads of these specimens were dissected; 5-mm samples were obtained and fixed in 2.5% glutaralde-hyde (Electron Microscopy Sciences) for six h;

afterwards, five 10-min washes were done us-ing Sorensen’s phosphate buffer. Post-fixation was performed with 1% osmium tetroxide for one h followed by buffer washes as described above. Then samples were dehydrated with a series of alcohols of increasing concentration (70% to 100%). Tissue samples were infiltrated in a propylene oxide solution and epoxy resin for three h and then, embedded in EPON 812 resin. Ultra thin 70-nm sections were obtained using an ultramicrotome, and these were stained with uranyl acetate and lead citrate. Fi-nally, sections were examined in a Jeol-100SX (60000 V) transmission electron microscope and micrographs from different portions of the gonad were obtained.
To characterize spermatogenesis, gametes in different development stages were selected, and a number of morphological parameters commonly used for comparative purposes were measured. In spermatogonia, spermatocytes (primary and secondary) and spermatids, the total and nucleus diameter were measured. In the mature spermatozoa, acrosome length (linear distance from the posterior edge of the acrosome to the posterior edge of the nuclear invagination), nucleus length (linear distance from the posterior edge of the invagination to the anterior edge of the midpiece) and nucleus width (at its widest point) were measured.

RESULTS

The male gonad of A. maura comprises blind tubules in which gametes are aligned along the inner wall. Spermatogenesis follows a concentric arrangement; from the inner wall towards the lumen, all gamete development stages co-occur in a single individual, includ-ing spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa. Cells of any given stage are arranged in clus-ters, and spermatozoa agglomerate toward the tubule center with flagella directed towards the lumen.

Spermatogonia

Spermatogonia are located along the inner wall of tubules, arranged in 2 or 3 cell rows. In the first layer, soermatogonia either are occa-sionally in contact or separated by cytoplasm projections of Sertoli cells. Spermatogonia measure 4 to 8 um and have irregular edges. The nucleus measures 2 to 5 um, with a

SPERMATOGENESIS IN ATRINA MAURA ail

heterogeneous chromatin arrangement, and contains one or two nucleoli (Fig. 1). The cyto-plasm includes numerous mitochondria, Golgi apparatus and occasional dense aggregates (Fig: 2):

Spermatocytes

Primary spermatocytes measure 3-4 um; the nucleus is similar in shape to that of the sper-

matogonia, but shows early signs of chromatin condensation. The cytoplasm is reduced, and thus the nucleus: cytoplasm ratio increases. The cytoplasm contains the same organelles as spermatogonia. Spermatocytes develop
cytoplasmic projections devoid of organelles.
Cell edges are irregular (Fig. 3).
Secondary spermatocytes are rarely ob-
served, probably due to the rapid onset of the
second meiotic division (Fig. 4). These cells

FIGS. 1-4. Transmission electron micrographs of spermatogonia and spermatocytes in Afrina maura males. FIG. 1: Espermatogonia (spg) in bands separated by pseudopodia-like projections (psc) of Sertoli cell (sc); the nucleus may display one or two nucleoli (ncl); FIG. 2: Spermatogonia surrounded by Sertoli cell (sc), the nucleus (п) is voluminous, 9: Golgi apparatus, m: mitochondria, da: dense ag-gregates; FIG. 3: Primary spermatocyte showing early chromatin condensation, g: Golgi apparatus, m: mitochondria, pp: pseudopodia-like cytoplasm projection; FIG. 4: Secondary spermatocyte showing heterochromatin condensation (dark area) in the nucleus towards the periphery.

332 CAMACHO-MONDRAGON ET AL.

are irregularly shaped and are dimensionally
similar to primary spermatocytes. The nucleus
is spherical and heterochromatin is condensed
towards the periphery. The cytoplasm is re-
duced and mitochondria are the most abundant
and visible organelles.

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Spermatids

Spermiogenesis (spermatid differentiation)
starts with spherical cells measuring 2.5 um in
diameter and that have a round nucleus with
scattered marginal heterochromatin (Fig. 5).

FIGS. 5-9. Transmission electron micrographs of spermatids in Afrina maura males. FIG. 5: Spermatids (spm) in early development stages; FIG. 6: Spermatids in advanced development stages; nucleus (п); mitochondria (m); proacrosomal vesicle (pav); FIG. 7: Mature spermatids. The proacrosomal vesicle is observed at different positions in its migration towards the cell apex; distal centriole (dc); proacrosomal vesicle (pav), flagellum (f); proximal centriole (pc), mitochondria (m); FIG. 8: Mature spermatids, acrosome (a), mitochondria (m); FIG. 9: Mature spermatids. Note the cell bridge (cb); distal centriole (dc); proximal centriole (pc), mitochondria (m).

SPERMATOGENESIS IN ATRINA MAURA 333

FIGS. 10-15. Transmission electron micrographs of mature sperm in Atrina maura
males. FIG. 10: The condensed nucleus is observed; acrosome (a), nucleus (n), mi-tochondria (m); FIG. 11: Detail ofthe acrosome that shows one electron-dense region
solid arrow) and one electron-translucent region (dotted arrow), nucleus (n); FIG. 12: The nucleus shows two invaginations: anterior invagination (ai) and posterior invagina-tion (pi); FIG. 13: Posterior view ofthe midpiece with four mitochondria (m) surrounding the distal centriole (*); FIG. 14: The box shows the nine microtubule triplets in the proxi-mal centriole (pc). Flagellum cross section (f) showing the internal 9 + 2 microtubular structure; FIG. 15: The distal centriole (dc) constitute the flagellum base.

334 CAMACHO-MONDRAGON ET AL.

The amount of cytoplasm decreases and sev-
eral clusters of mitochondria are observed. As
development progresses, the nucleus becomes
smaller, and chromatin becomes scattered
in irregular clumps. In this stage, spermatids
display four voluminous mitochondria that congregate at the posterior side of the nucleus. A proacrosomal vesicle appears in the same zone, which shows a characteristic “V”-shaped arrangement of electron-dense material to the center (Fig. 6). The cytoplasm displays two centrioles in orthogonal position exhibiting the typical nine triplets of microtubules. The fla-gellum is produced by the distal centriole, the closest to the plasma membrane (Fig. 7). Mature spermatids are characterized by the condensation of nuclear material that begins at the periphery. The proacrosomal vesicle migrates from the bottom to the apex of the cell (Fig. 7) and becomes conical, being invagi-nated and less electron-dense at the periphery (Fig. 8). Spherical mitochondria are located at the base of the nucleus and surround the centrioles. The Golgi apparatus is commonly not observed. Spermatids may be connected by intracellular bridges that link the bottom of one cell with the apex of another (Fig. 9).
In summary, spermiogenesis involves the progressive condensation of chromatin from the periphery to the center, coupled with the

reduction of the nucleus size; the formation of a proacrosomal vesicle at the base of cells and its migration to the apex, where it becomes the acrosome; the fusion of small mitochondria into four mitochondrial spheres arranged around centrioles; the production of the flagellum by the distal centriole; the reduction of cytoplasm into a very thin layer and the loss of the Golgi apparatus.

Spermatozoa

Mature spermatozoa has a conical head measuring ~ 2 um from the acrosome tip to the back of the midpiece. The nearly spheri-cal nucleus is fully condensed and measures ~ 1.4 um long by ~ 1.6 um wide (Fig. 10). A small conical acrosome (~ 0.7 um, includ-ing subacrosomal material), without an axial rod, is located to the front. The acrosome is invaginated in the posterior end. It appears to be composed of two different materials, one electron-dense outer region shaped as a thin elliptical line and an electron-translucent inner region. The subacrosomal space contains
granular material (Fig. 11).
The nucleus shows two invaginations: one
to the front, adjacent to the acromosome,
containing moderately dense granular material,
and the other to the back, where the proximal

FIGS. 16, 17. Transmission electron micrographs of Sertoli cells in Atrina maura males. FIG. 16: One Sertoli cell (sc) is observed adjacent to the first spermatogonia (spg) row. The Sertoli cell content includes lipid droplets (*) and small glycogen granules (arrow); FIG. 17: Detail of one Sertoli cell in contact with three spermatogonia.

SPERMATOGENESIS IN ATRINA MAURA 339

centriole is located (Fig. 12). At the base of the nucleus, mitochondria are located near the nucleus membrane. Up to four spherical mitochondria measuring 0.5 um in diameter were observed surrounding the diplosoma, which constitutes the midpiece (Fig. 13). The proximal centriole is located at the posterior invagination of the nucleus perpendicular to the axoneme and shows a configuration of nine microtubule triplets (Fig. 14). The distal centriole constitutes the basal body of the flagellum (Fig. 15), which displays the typical 9 + 2 structure of inner microtubules (nine peripheral microtubules surrounding a central pair) (Fig. 14). The spermatozoa cytoplasm is highly reduced, so that the nucleus : cytoplasm ratio is high in these cells.

Sertoli Cells

Inside the tubules large irregular-shaped so-matic cells known as Sertoli cells can be found. The cytoplasm of these cells contains a large number of lipid droplets and small dispersed glycogen particles (- 0.5um) (Figs. 16, 17). No membrane-enclosed organelles were observed in the cytoplasm of these cells.

DISCUSSION

The general spermatogenesis process in A. maura is similar to that of other bivalves (Thielley et al., 1993; Gaulejac de et al., 1995; Chung et al., 2007). In any one specimen, gam-etes in all development stages were observed displaying the concentric distribution that char-acterizes the male gonad of this species (Ca-macho-Mondragön et al., 2012). However, the different cell types were not observed with the same frequency. Specifically, secondary sper-matocytes were rarely observed; this condition has been reported previously for other bivalves and is attributed to the rapid cell division rate, which is difficult to distinguish histologically using light microscopy (Sastry, 1979).
The major changes in cell morphology during the spermatogenesis of A. maura occur in the spermatid stage, including the condensation of nuclear chromatin, the mitochondrial fusion and acrosome formation. As in P. nobilis and A. pectinata, also members of the family Pinnidae (Gaulejac de et al., 1995; Kang et al., 2012), in A. maura the proacrosomal vesicle and the flagellum appear until the early spermiogenesis

stages, in contrast to bivalve species such other as Barbatia obliquata (W. Wood, 1828) and B. foliata (Forsskal in Nieburh, 1775) (Arcidae), Brachidontes semistriatus (Krauss, 1848) (Mytilidae), Arcuatula capensis (Krauss, 1848) (Mytilidae), Bathymodiolus childressi Gustafson, Turner, Lutz & Vrijenhoek, 1998 (Mytilidae) and Tivela polita (С. В. Sowerby Il, 1851) (Veneridae), all of which show an early formation of both structures in spermatogonia and spermatocytes (Reunov & Hodgson, 1994; Eckelbarger & Young, 1999). In A. maura, the acrosome is formed at the posterior end of the spermatid and subsequently migrates to the anterior end, as in other bivalves (Erkan & Sousa, 2002; Chung et al., 2007). When this migration is completed, the acrosome shows an outer apical region that is more electron dense and homogeneous relative to the electron-translucent inner region. This differ-ence in electron density across the acrosome has been observed in Perna perna (Linnaeus, 1758) (Bernard & Hodgson, 1985), Pinctada margaritifera (Linnaeus, 1758) (Thielley et al., 1993), Chama macerophylla Gmelin, 1791, and Spisula solidisima (Dillwyn, 1817) (Hylander & Summers, 1977); this may be due to the various functions of the acrosome during fertilization (Popham, 1974). There is evidence that the electron-dense region is involved in the binding of spermatozoa to the microvilli on the oocyte surface, releasing lysines during the acrosome reaction (Hylander & Summers, 1977), maybe arylsulphatase and acid phosphatase as stated by Pipe & Da Silveira (1989) for Mytilus edulis Linnaeus, 1758. Whereas, the electron-trans-lucent region might result from the crystalline alignment of enzymes involved in fertilization (Friend & Fawcett, 1974).
Numerous studies have demonstrated that all bivalves have “primitive” spermatozoa (Fran-zen, 1956) or aqua sperm (Rouse & Jamieson, 1987), typical of animals that release their gametes to the surrounding aquatic environ-ment. Rouse & Jamieson (1987) recognize two aqua-sperm categories: (1) ect-aquasperm: spermatozoa that, at least potentially, fertil-izes oocytes in the surrounding water, (2) ent-aquasperm: spermatozoa released to water but that is subsequently attracted by an incoming current to the mantle cavity where fertilization takes place. In the case of A. maura, the spermatozoa displays the basic structural plan, presumably suitable for swimming (ect-aquasperm), with an acrosomal complex, a

336 CAMACHO-MONDRAGON ETAL.

condensed spheroidal nucleus, a midpiece
consisting of four mitochondria surrounding
the two centrioles and a flagellum with a 9 + 2
microtubular configuration.
The morphology of the midpiece in A. maura
is similar to the one reported for several bi-valves (Popham, 1979): four mitochondria are apparent, similar to the spermatozoa of other members of the order Pterioidea (Healy, 1989). These giant mitochondria likely result from the fusion of small preexisting ones, as reported for other bivalves (Longo & Dornfeld, 1967; Bernard & Hodgson, 1985; Hodgson & Bernard, 1986). Their position around the flagellum suggests that they may be involved in the energy supply for spermatozoa mobility and metabolism.
The shape of the nucleus, which is basically the mature spermatozoa head, varies from one mollusk species to another. In the case of the “primitive” bivalve sperm, the nucleus shape may be either ovoid, conical or spheri-cal (Popham, 1979). A. maura’s spermatozoa has a spherical nucleus that exhibits a granu-lar pattern of chromatin condensation during spermiogenesis, similar to other bivalves with nearly spherical spermatozoa nuclei (Maxwell, 1983).
Unlike spermatozoids from other mollusks (e.g., gastropods), bivalve spermatozoids pos-sesses a limited number of available features for taxonomic or phylogenetic purposes. Traits which provide relevant information include the shape and inner structure of the acrosomal vesicle, and the spatial relationship between this vesicle and the nuclear apex (Healy et al., 2000). The nucleus of spermatozoids in A. maura has an anterior invagination under the acrosomal vesicle that contains a substance of unknown nature which is not organized in an axial rod (or perforatorium); the same is observed in A. vexillum and apparently repre-sents the primitive bivalve condition (Popham, 1979). Moreover, the absence of an axial rod and the presence of a small acrosomal vesicle (~ 0.7 um) in A. maura may be associated with functional aspects and be related to the thick-ness of the vitelline membrane. In the family Mytilidae, for example, there is a well-defined axial rod and a long acrosomal vesicle (2 um), and both traits are attributed to the fact that in this family oocytes have a thicker membrane (Healy et al., 2000).
The ultrastructural details of the A. maura spermatozoa observed in this study essentially

agree with those reported for other members of the family Pinnidae, such as P. nobilis (Gaulejac de et al., 1995), A. vexillum (Healy et al., 2000) and A. pectinata (Kang et al., 2012), but also contribute additional informa-tion regarding the acrosomal vesicle profile and substructure. These allow the identifica-tion of significant morphological differences and similarities between species in the family. Four important features were detected in A. maura: (1) the acrosomal vesicle is conical and larger (length - 0.7um) than that reported for A. vexillum (~ 0.4 um) and A. pectinata (~ 0.5 um), deeply invaginated at the base and containing material differentiated in a highly electron-dense anterior layer forming a thin elliptical line (which might correspond to the radiated plates in A. vexillum) and a less electron-dense posterior layer. This particular arrangement of electron-dense material (in a thin elliptical line) in the acrosomal vesicle was also detected in A. vexillum (Healy et al., 2000) and A. pectinata (Kang et al., 2012), but not in other species of the families Pteriidae, Ostreidae, Mytilidae and Arcidae (Healy et al., 2000), so this feature could be used for taxonomic analyses at the family level; (2) the subacrosomal material is highly granular and, although it fills the nuclear depression, does not spread to the upper limit of the vesicle invagination as in A. vexillum; (3) the nucleus is small (~ 1.4 um) compared with the large nuclei (2-10 um) reported for A. vexillum and species of other families such as Arcidae and Glycymeridae (Healy et al., 2000). It also displays a granular texture and a remarkable anterior invagination; (4) mitochondria are spherical and are associated with the centriole pair in the three species; however, A. maura presents four in contrast with five commonly observed in Р nobilis, A. vexillum and А. pec-tinata. Healy (1995) reported that the number of mitochondria in the spermatozoa midpiece is a trait that is widely used in taxonomic analyses, since it tends to be stable at the family or superfamily level. Recently, has been reported that the families Arcidae, Mytilidae, Pteriidae and Pinnidae of the subclass Pte-riomorphia all have spermatozoids with five mitochondria (Healy et al., 2000; Kang et al., 2012). However, it is accepted that the number of mitochondria in the midpiece may vary to some extent between species in the same family (Healy et al., 2000). Sometimes, this trait may display slight variations even within

SPERMATOGENESIS IN ATRINA MAURA Sor

a single species (Healy, 1995), although A. maura always displayed four mitochondria. On the other hand, the use of electron mi-croscopy techniques has allowed us to unveil the details regarding the evolution of various cell categories and the presence of specific somatic cells that accompany the development of bivalve gametes (Avendaño & Le Pennec, 1998). In A. maura, the presence and relative abundance (in accordance with the gonad ripening) of adipogranular cells in the male gonad was reported by Camacho-Mondragön et al. (2012) using only the paraffin histological technique coupled with Sudan black staining to demonstrate the lipid content. The present investigation could not confirm the existence of these cells, but instead revealed the presence of Sertoli cells inside the tubules.
The arrangement of Sertoli cells in the male gonad of A. maura could not be described in detail previously due to the intrinsic limitations of light microscopy. The higher resolution of the present study revealed that Sertoli cells only surround spermatogonia, that is, any interaction of Sertolli cells with any other gamete type ina more advanced developing stage was not ob-served. This finding reinforces the hypothesis of the active role of Sertoli cells in nutrition during the early stages of spermatic development, as observed in other bivalve species (Pipe, 1987; Mathieu & Lubet, 1993; Eckelbarger & Young, 1999). The cytoplasm of Sertolli cells includes a number of biomolecules, such as protein, glycogen and lipid granules, all of which are transferred to gametes through pinocytosis to support the metabolic reactions required in this stage (Herlin-Houtteville & Lubet, 1975; Pipe, 1987).

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338 CAMACHO-MONDRAGON ETAL.

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